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ORIGINAL ARTICLE

Modified method of AgNOR staining for tissue and interpretation in histopathology

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Summary

This study was conducted in the department of Pathology King Edward Medical University, from June to December 2002 to introduce the new method of AgNOR staining and its interpretation to increase its reliability. A total of 60 brain specimens were stained with modified AgNOR technique. The diagnosis of malignancy was made on H & E staining. AgNOR counts, variation in size and dispersion of AgNOR dots in cells were graded and compared in malignant and non-malignant lesions. Modified method of AgNOR staining and interpretation was an easy, reliable and reproducible alternative to traditional AgNOR techniques for evaluating proliferation activity of cells in malignant and benign brain lesions. mAgNOR counts of different grades of astrocytoma (2.97 ± 0.96, 3.97 ± 0.43, 6.01 ± 2.74 and 8.01 ± 3.56) were significantly ($P < 0.01$) greater when compared with counts of normal brain (0.40 ± 0.01), and reactive gliosis (0.60 ± 0.01). AgNOR size and dispersion were of higher grade in a significantly greater proportion of malignancy when compared with benign conditions ($P < 0.05$). The AgNOR dots were brighter and more clear with modified staining when compared with previous studies. We conclude that modified AgNOR staining technique is simple, quick and reliable to evaluate cell proliferation by detecting AgNORs size and dispersion. In future, AgNOR size and dispersion should be considered rather than the count only. We recommend the use of morphometry for AgNOR size in future. We also recommend the use of modified AgNOR staining for obtaining sound and confidant results in routine paraffin sections.

Keywords
AgNOR, astrocytoma, astrogliosis, glioblastoma multiform, gold chloride, neutral red, sodium thiosulphate

The nucleolar organizer regions (NORs) are chromosomal loops of DNA involved in ribosomal synthesis (Gall and Pardue 1969). Associated with NORs there are some nucleolar proteins, which are stained with silver methods (AgNOR proteins or AgNORs) (Derenzini and Ploton 1991). AgNORs can be identified as black dots in the nuclei. Their size and number reflect nucleolar and cell proliferative activity of tumours (Derenzini *et al.* 1990).

A simple silver staining technique (AgNOR method) (Pich *et al.* 1995) can easily detect AgNORs in formalin-fixed, paraffin embedded tissues and permits a rapid evaluation of morphology and tumour cell kinetics even on small biopsies. Estimation of AgNORs parameters (number, size and distribution) has been applied in tumour pathology both for diagnostic and prognostic purposes. AgNOR number and distribution in the nucleus (configuration) were useful in the detection and prognosis of some neoplasias, such as renal, bladder, and pharyngeal carcinoma, multiple myeloma, and skin melanocytic lesions (Pich *et al.* 1995).

AgNOR analysis in multiple myeloma allowed the identification of atypical plasma cells with different proliferative activity in bone marrow biopsies (Pich *et al.* 1992). It was helpful in the classification of urothelial carcinoma in different grades of malignancy and in subdividing patients with grade II transitional cell carcinomas into low- and high-risk groups (Helpap *et al.* 1994; Pich *et al.* 1994).

Typing of AgNOR size and dispersion was also found to be an easy and reproducible alternative to traditional AgNOR counts for differentiating malignant from non-malignant effusions. These parameters should be correlated with the already established but expensive techniques of AgNOR area and size imaging by electron microscopy and flow cytometry, as an economical alternative (Khan *et al.* 2006).

There are different methods of AgNOR staining. One of these was originally introduced by Ploton *et al.* (1986), who tried to improve the staining by using as toning agent 10% sodium thiosulphate without any counterstain. Another method was adopted by Mourad *et al.* (1993, 1997), who used 1% gold chloride as a toning agent, and slides were counterstained. Banacroft has mentioned a different method and recommended light counter stain and 1% gold chloride as toning agent (Banacroft 2002).

In the present study, we adopted both methods and compared their results. The objective was to compare the different staining techniques used for AgNOR and to determine the best method.

Patients and methods

We selected 60 samples, 10 samples each of normal brain tissue, reactive gliosis, pilocytic astrocytoma, astrocytoma grade II, astrocytoma grade III, and glioblastoma multiform (grade IV astrocytoma). We performed the AgNOR staining with previously adapted procedure and modified procedures. In previous methods counter stain (Neutral red) was used. We compared the results both with and with out counter stain with both tonic agents (gold nitrate, sodium thiosulphate) (Table 1).

Preparation of solutions

Solution A The solution was prepared by dissolving 500 mg gelatin powder in 25 ml deionized water at 37 °C and then 250 μl formic acid is added. Continuous shaking for about 10 min at 37 °C was sufficient to dissolve the gelatin and a clear solution was obtained.

Solution B It comprised silver nitrate and deionized water. Small-capped bottles were used in which different quantities of silver nitrate like 1, 2, 3 or 4 g were kept. As much as

Table 1 Reagents required for modified AgNOR procedure

Serial number	Name of reagents	Amounts of reagents	Concentration	Comments
	Solution A		2%	
	Gelatin powder	500 mg		
	Formic acid	250 μl		
	Deionized water	25 ml		
	Solution B		50%	
	Silver nitrate	30 g		It is very costly and use the amount required for the number of cases
	Deionized water	60 ml		
	Working solution			Make just before use
	Solution A	1 part		
	Solution B	2 parts		
	toning solution	Gold chloride	1%	Very clear identification of AgNORs
		Sodium thiosulphate	10%	Gives good results but less clear when compared with 1% gold nitrate
	Counter stain	Neutral red	Very light	Should not be used because of the blurring of AgNOR staining

50% w/v concentrated solution of silver nitrate in deionized water was prepared just before staining because it is very costly, only the exact amount required for the number of cases to be stained was used.

Working solution

This was prepared by mixing one part by volume of solution A with two parts by volume of solution B and it was filtered through filter paper into plastic bottle and used immediately because it degrades immediately. The quantity of silver nitrate adequate to stain the number of slide in a particular batch was used. Two grams of silver nitrate and hence 6 ml of final working solution were found sufficient to stain five sections.

Haematoxylin and eosin staining

All the sections were stained for Haematoxylin and eosin by two competent histopathologists using the technique by Bukhari *et al.* (2004) to confirm the lesions, normal tissue and reactive gliosis.

The AgNOR stain

Four-micron unstained sections were obtained from the selected areas of tissue to perform the silver staining. The tissue was deparaffinized in several changes of xylene and descending alcohol concentrations. Rehydration was then performed in several changes of ultrapure distilled water. The tissue was then incubated in acid alcohol (three parts ethanol: two parts acetic acid) for 5 min and then rinsed in ultra pure distilled water several times. The sections were then incubated with silver nitrate solution in a dark humidified chamber for 38 min at room temperature 37 °C. The silver staining solution consisted of two parts of a 50% solution of silver nitrate and one part 2% gelatin in 1% formic acid solution. Ultra pure distilled water was used for preparation of all solutions.

Some of the sections were then incubated in 1% solution of gold chloride and other sections with a 10% solution of sodium thiosulphate for 5 min. The sections were then washed in distilled water, dehydrated in graded alcohol and then xylene and cover slipped. The tissue was then ready for counts. Neutral red counter stain was used for few slides to see the contrast, while for others no counter stain was used.

AgNOR Stained section was examined under the light microscope (Olympus CHT, Optical.Co.Ltd, Japan) using $4 \times 10 \times 40 \times 100\times$, Objective and eyepieces of $10\times$ giving a maximum magnification of 1000.

The condenser was adjusted to change light intensity and allow test visualization of the AgNORs. The slide of astrocytoma was scanned with $4 \times 10\times$ and $40\times$ objectives. AgNOR counting was performed under $100\times$ using oil immersion. The nuclei stain light yellow and out line of nuclei as well as cells were usually clearly visible. The AgNORs were visualized as brown black discrete dots of variable size within the nuclei. AgNOR in 100 cells was counted and size variation and distribution were also recorded.

AgNOR counts, size and distribution

Two investigators without knowledge of the method of AgNOR stain, tumour type, grade, stage, or disease outcome, performed the AgNOR counts, size and distribution. Two counts were performed. The first count was the mean number of AgNORs in 100 tumour nuclei (mAgNOR).

The second count was the percentage of nuclei exhibiting five or more AgNOR granules/nucleus/100 cells called proliferative index (pAgNOR). This count was believed to represent proliferative activity. Tumours having a pAgNOR count of 8% or more were considered to display high proliferative activity. (Mourad *et al.* 1997).

The grading of size variation was performed according to Khan *et al.* (2006) and scores of distribution are given as below: 0, more or less uniform in size; 1+, two different sizes; 2+, more than two different sizes (but not those of 3+); 3+, including all grades and sizes.

The grading of dots dispersion was performed according to Khan *et al.* (2006) and scores of dispersion of AgNOR dots are given as below: 0, limited to nucleoli; 1+, occasional dispersion outside nucleoli; 2+, moderate dispersion outside nucleoli; 3+, widely dispersed through the nucleus.

Statistics

Mean AgNOR count and standard deviation of each mean were calculated (as mAgNOR). The AgNOR Proliferative index (pAgNOR) were calculated by counting the cells having five or more AgNOR granules per nucleus, in 100 Nuclei. '*t*' test was applied to find the probability between the means of different groups.

Results

There was very good contrast of AgNOR after toning with 1% solution of gold nitrate or with a 10% solution of sodium thiosulphate when compared with un-toned section. The results of 1% gold chloride were more reliable when compared with 10% solution of sodium thiosulphate.

Modified method of AgNOR staining for tissue

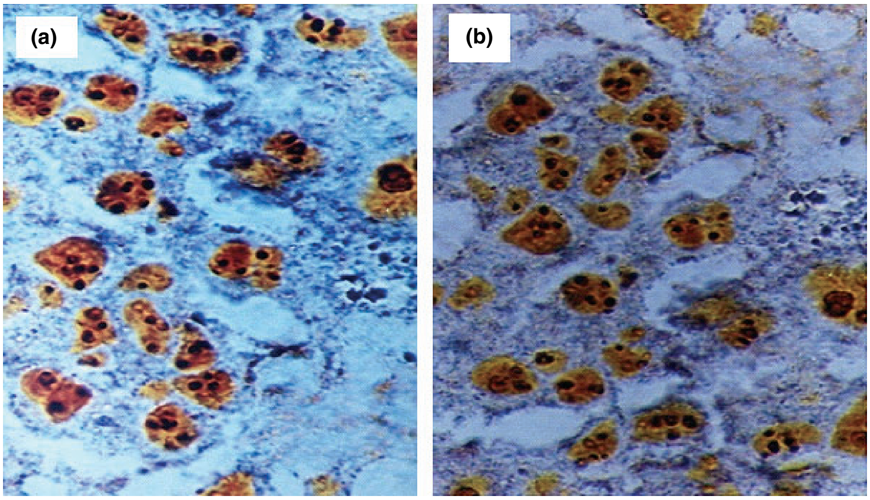

Figure 1 (a) Photomicrograph showing AgNORs in grade II astrocytoma without counterstain and toning with sodium sublimate: AgNOR dots size and dispersion are clearly visible. (b) Photomicrograph showing another section of same grade II Astrocytoma with 10% sodium thiosulphate but with counter stains. The AgNOR dots have become blurred due to dim background and less clearly visible as compared to photomicrograph (a).

AgNORs dots size and dispersion were clearer in sections where no counterstain was used when compared with counterstained sections with neutral red (Figures 1 and 2).

AgNOR interpretation

Count in normal, reactive and malignant astrocyte The AgNORs appeared as black discrete dots in a pale yellow stained nucleus of astrocytes. The mAgNOR (mean AgNOR count) and pAgNOR (percentage of AgNOR dots) counts were significantly high in different grades of astrocytoma, while almost at normal level in normal brain tissue and in reactive gliosis (Table 2).

Aneuploidy was indicated when tumours had mAgNOR counts of 2.4 or more. These counts were high in all 40 cases of astrocytoma of grades I–IV and normal in normal brain tissue (0.40 ± 0.01) and mild in reactive gliosis (0.60 ± 0.01).

The mAgNOR count in astrocytoma increased from grades I to IV. It was 2.97 ± 0.96 in pilocytic astrocytoma, 3.97 ± 0.43 in astrocytoma grade II, 6.01 ± 2.7 in astrocytoma grade III and 8.01 ± 3.56 in astrocytoma grade IV (glioblastoma multiform). This difference is significance. ($P < 0.05$). pAgNOR counts were high in gradually increasing astrocytoma, 25% in pilocytic astrocytoma, 50% in all astrocytoma grade II, 65% in all grade III and 72% in all

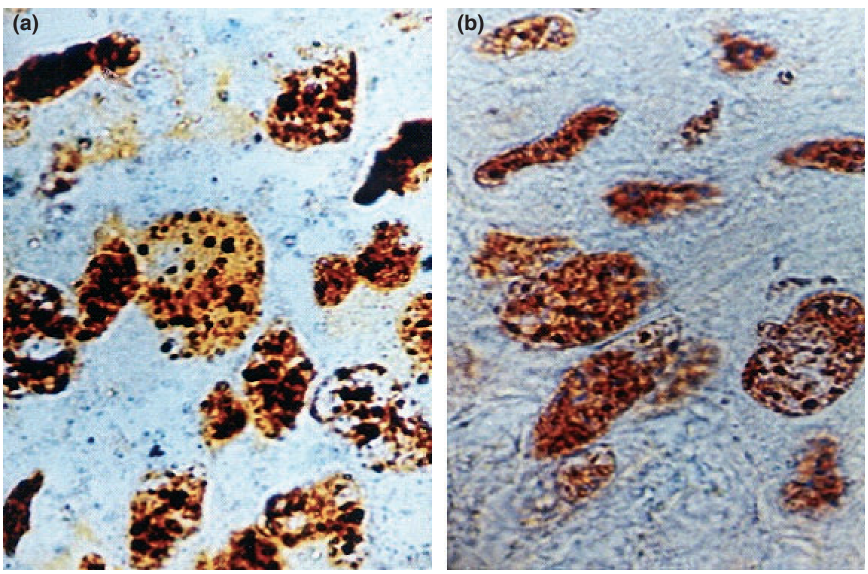

Figure 2 (a) Photomicrograph showing AgNORs in grade IV Astrocytoma without counterstain and toning with 1% Gold chloride: AgNOR dots size and dispersion are clearly visible. (b) Photomicrograph showing another section of same grade IV Astrocytoma with 1% gold Chloride but with counter stains. The AgNOR dots have become blurred due to dim background and less clearly visible when compared with photomicrograph (a).

Table 2 Comparison of mean AgNOR count, size, and distribution per cell between in different lesions

Lesions	Number of cases	AgNOR count		AgNOR variation in size per cell (mean ± SD)	AgNOR dispersion per cell (mean ± SD)
		mAgNOR count per cell (mean ± SD)	pAgNOR Proliferative Index (percentage of nuclei with ≥5 AgNOR dots/nucleus		
Normal brain tissue	10	0.40 ± 0.01	5%	0.20 ± 0.15	0.18 ± 0.68
Reactive gliosis	10	0.60 ± 0.01	8%	0.23 ± 0.23	0.20 ± 0.54
Pilocytic astrocytoma	10	2.97 ± 0.96	25%	0.25 ± 0.19	0.29 ± 0.13
Astrocytoma grade II	10	3.97 ± 0.43	50%	0.45 ± 0.18	0.43 ± 0.24
Astrocytoma grade III	10	6.01 ± 2.74	65%	1.24 ± 0.26	0.93 ± 0.42
Astrocytoma grade IV	10	8.01 ± 3.56	72%	1.86 ± 0.80	1.56 ± 0.44

grade IV astrocytoma. It was <5% in normal brain tissue and 8% in reactive gliosis. This count was believed to represent proliferative activity. Tumours having a pAgNOR count of 8% or more were considered to display high proliferative activity. The AgNOR count in malignant cells was significantly higher than that in resting cells and reactive cells ($P < 0.01$), and the count significantly increased with tumour grade ($P < 0.01$). AgNOR count of resting cells was close to reactive cells ($P > 0.05$).

AgNOR dots size in normal, reactive and malignant astrocyte Variation in AgNOR size in grades I and II astrocytoma was also observed. There was non-to mild variation in the size of AgNOR dots in low grade astrocytoma (mean 0.25 ± 0.19). There was moderate to high variation in the size of AgNOR dots in high grade astrocytoma (mean 1.86 ± 0.80). The statistical difference between the variations of AgNOR dot size was significantly high between low grade and high grade astrocytic glioma ($P < 0.05$).

AgNOR dots dispersion in normal brain tissue, reactive gliosis and different grades of astrocytoma The mAgNOR and pAgNOR counts were significantly high in different grades of astrocytoma. This difference was significantly high between low and high-grade astrocytoma ($P < 0.05$). The mean dispersion of AgNOR per cell in normal tissue and reactive gliosis was nil, while low in low-grade astrocytoma and high in high-grade astrocytoma (mean 1.56 ± 0.44 in grade IV). The difference was significant between low and high-grade astrocytoma ($P < 0.05$) (Table 2).

Discussion

Our results show that the mAgNOR was 2.97 ± 0.96 in pilocytic, 3.97 ± 0.43 in astrocytoma grade II, 6.01 ± 2.74 in astrocytoma astrocytoma grade III and 8.01 ± 3.56 in astrocytoma grade IV (glioblastoma multiform). The difference is significant ($P < 0.05$). pAgNOR counts were high in gradually increasing astrocytoma: 25% in pilocytic astrocytoma, 50% in all astrocytoma grade II, 65% in all grade III and 72% in all grades IV astrocytoma. It was <5% in normal brain tissue and 8% in all-reactive gliosis. The AgNOR count in malignant cells was significantly higher than that in resting cells and reactive cells ($P < 0.01$), and the count significantly increased with tumour grade ($P < 0.01$). AgNOR count of resting cells was close to reactive cells ($P > 0.05$). Regarding the reactive gliosis, no doubt that a pAgNOR value of 8% indicates a high proliferative activity, but the size and dispersion of AgNORs are normal. These results are consistent with the findings of Khan *et al.* (2006) who concluded that typing of AgNOR size and dispersion is more reliable and reproducible alternative to traditional AgNOR counts for differentiating malignant from non-malignant effusions.

Our results are in accordance with studies on bladder carcinoma and multiple myeloma showing association between mean AgNOR count and histological grade of differentiation(Pich *et al.* 1992, 1994) and with a study on oral lesions showing that the mean AgNOR count in papilloma was higher than in the hyperplasia, and lower than in the well- and poorly differentiated oral squamous cell carcinomas (Fonseca and do Carmo 2000).

The main finding of our study is that AgNOR size and dispersion remained low in normal reactive gliosis while high in malignant tumours. The results are in accordance with previous reports in multiple myeloma (Pich *et al.* 1992, 1995), bladder carcinoma (Pich *et al.* 1992, 1995; Helpap *et al.* 1994), and skin melanocytic lesions (Pich *et al.* 1995) showing that AgNOR dispersion is high in less differentiated tumours. AgNOR size and dispersion may be more important than counting alone: indeed AgNOR count may increase also in reactive conditions, while size and dispersion changes are seen only in malignant conditions, as has been concluded by other investigators (Akhtar *et al.* 2004; Khan *et al.* 2006).

In our study, the mAgNOR and pAgNOR (proliferative index) were high in more proliferative and mitotically active tumours cells as we moved up in different grades of astrocytoma (i.e. from G-I to G-IV). We therefore proposed that mAgNOR would probably be the reflection of the total number of chromosomes or ploidy, and the percentage of cells with five or more AgNOR granules in 100 cells (pAgNOR) would reflect proliferative activity, as already reported by Mourad *et al.* (1993). In this study, we also found that pAgNOR is more predictive of aggressive behaviour in astrocytoma grade III and glioblastoma multiform than mAgNOR. These findings are consistent with other reports of Helpap *et al.* (1994), Khan *et al.* (2006), Hashmi *et al.* (2006) and Parveen *et al.* (2006).

In a standardized morphometric analysis of AgNORs in breast carcinoma it was found that a significant inverse correlation existed between proliferation markers and oestrogen/progesterone receptor status and histopathological grade. AgNOR expression was significantly higher in cycling (MIB1 positive) tumour cells, than in resting (MIB1 negative) ones, however with certain exceptions. It was concluded, that standardized AgNOR parameters correlate with markers of increased malignant potential in breast carcinomas. However, AgNORs seem to reflect proliferation independent of cellular and nucleolar activity of tumour cells, as well (Bankfalvi *et al.* 1998; Tomobe *et al.* 2001). These findings are consistent with our results and suggestions that size and configuration of the dots are more important for interpretation of the results in malignant neoplasms.

We are aware that morphometric quantitative analysis for AgNOR dots should be performed; however, the technique is rather expensive and, unfortunately, is not available in our laboratory. On the contrary, the parameters of size and configuration may become an easier, cheaper and accurate method of estimation of proliferative index that is an important prognostic factor for most malignant neoplasms. Lastly, the findings of this modified method of staining using 1% gold chloride and 10% sodium sublimate proved better in evaluating all AgNOR parameters. These findings are not consistent with those reported by Mourad *et al.* (1993) and Banacroft (2002). The staining results of our study were brighter with toning agents as consistent with Mourad *et al.* (1997), but results with 1% gold chloride are brighter than the results with 10% sodium thiosulphate. Our findings are contradictory to those reported by Banacroft (2002) who used counterstain for AgNORs. In our study, the results of size and dispersion of AgNORs were clearer without the use of counterstain. This method saves time, cost and gives far better results.

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